



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵: C07K 15/28, C12P 21/06, A61K 39/395, C12N 15/00, 1/00, 5/00, C07H 15/00	A1	(11) International Publication Number: WO 94/14847 (43) International Publication Date: 7 July 1994 (07.07.94)
(21) International Application Number: PCT/US93/12339 (22) International Filing Date: 17 December 1993 (17.12.93) (30) Priority Data: 07/996,675 24 December 1992 (24.12.92) US (71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 300 Lakeside Drive 22nd floor, Oakland, CA 94612 (US). (72) Inventors: ZANETTI, Maurizio; 6112 La Jolla Hermose Avenue, La Jolla, CA 92037 (US). BILLETТА, Rosario; 359 Coast Boulevard South, La Jolla, CA 92037 (US). (74) Agents: DREGER, Walter, H. et al.; Flehr, Hobbach, Test, Albritton & Herbert, 4 Embarcadero Center, Suite 3400, San Francisco, CA 94111-4187 (US).		(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: GENETICALLY ENGINEERED IMMUNOGLOBULINS (57) Abstract This invention relates to the introduction of oligopeptide epitopes of biological receptor, preferably herein the CD4 receptor, for expressing within the three dimensional fold of an immunoglobulin (Ig) molecule, thus creating molecules useful to induce specific, biologically active anti-receptor immunity.		

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GENETICALLY ENGINEERED IMMUNOGLOBULINS

Cross Reference to Related Applications

This is a continuing application of applications U.S. Serial No. 07/947415 and U.S. Serial No. 07/947521, both
5 filed 18 September 1992, each being a continuation application of application U.S. Serial No. 07/316144, filed 24 February 1989.

Field of the Invention

The present invention may utilize in its preferred
10 embodiments, the use of recombinant DNA technology to genetically engineer natural or synthetically-derived immunoglobulin molecules, imparting therein novel epitopes, so as to create novel entities that can be employed *in vitro* and *in vivo* in a variety of means, such
15 as to immunize against pathogens, and for example, build tolerance to antigens.

In preferred embodiments, the epitopes are inserted into the so-called heavy or light chain variable domain of a given immunoglobulin molecule. Thus, known recombinant
20 DNA technologies come to bear in the present invention, helping create novel immunoglobulin entities that retain functionality by localizing to particular cell types mechanistically via the so-called constant domains but otherwise functionally exploited to provide a novel

localization of a particular antigenic determinant or epitope.

Background of the Invention

Recombinant DNA technology has reached the point currently
5 of being capable, in principle, of providing the
methodology sufficient to identify, isolate and
characterize DNA sequences, configure them for insertion
into operative expression vectors and transfect those
vectors variously into recombinant hosts such that those
10 hosts are harnessed in their ability to produce the
polypeptide encoded by the DNA sequence. Obviously, many
variations attend the methodology associated with
recombinant DNA technology, and particular means are not
without inventive faculty. Nonetheless, methods are
15 generally known in the published literature enabling
requisite mental equipment for the art skilled to practice
recombinant DNA technology in the production of
polypeptides from a given recombinant host system.

Immunoglobulins (Igs) are the main effectors of humoral
20 immunity, a property linked with their ability to bind
antigens of various types. In view of the myriad numbers
of antigens to a particular host organism, it can be
appreciated that there are a like number or more of
immunoglobulins that contain antigenic determinants or
25 epitopes against particular such antigens.

Immunoglobulin molecules are unique in their functionality
of being capable of localizing to certain cell types,
probably by means of mutual recognition of certain
receptors that are located on the cell membrane.
30 Immunoglobulins demonstrate a second general property
whereby they act as endogenous modulators of the immune
response. Igs and their idiotypic determinants have been
used to immunize at the B- and/or T-cell level against a
variety of exogenous antigens. In many cases, the

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immunity they evoke is comparable with that induced by the antigen itself. Although the principle underlying this phenomenon is understood, little is known about the molecular basis and the minimal structural requirements for the immunogenicity of Igs molecules and the interaction between those regions which may be responsible for such immunogenicity and the regions that are thought to provide the localization of a given immunoglobulin molecule with a particular cell/receptor type.

- 10 In the last many years, much progress has been made in endeavors to understand the immunogenic properties, structure and genetics of immunoglobulins. See Jeske, et al., Fundamental Immunology, Paul, ed., Raven Press, New York (1984), p 131 and Kabat, Journal Immunology 141, 525 (1988).

Initially, the antigenicity of the so-called variable (V) domain of antibodies was demonstrated. Oudin, et al., Academy of Sciences D 257, 805 (1963) and Kunkel, et al., Science 140, 1218 (1963). Subsequently, further research pointed out the existence of discrete areas of variability within V regions and introduced the notion of hypervariable (HV) or complementarity-determining regions (CDR). Wu, et al., J. Exp. Med. 132, 211 (1970). Many studies since have indicated that the immunogenic property of Ig molecules is determined presumably primarily by amino acid sequence contained in the CDRs. Davie, et al., Ann. Rev. Immunol. 4, 147 (1986).

The basic immunoglobulin or antibody structural unit is well understood. The molecule consists of heavy and light chains held together covalently through disulfide bonds. The heavy chains are also covalently linked in a base portion via disulfide bonds and this portion is often referred to as the so-called constant region which is thought responsible for a given immunoglobulin molecule being mutually recognizable with certain sequences found

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at the surface of particular cells. There are five known major classes of constant regions which determine the class of the immunoglobulin molecule and are referred to as IgG, IgM, IgA, IgD and IgE. The N-terminal regions of the so-called heavy chains branch outwardly in a pictorial sense so as to give an overall Y-shaped structure. The light chains covalently bind to the Y branches of the two heavy chains. In the regions of the Y branches of the heavy chains lies a domain of approximately 100 amino acids in length which is variable, and therefore, specific for particular antigenic epitopes incidental to that particular immunoglobulin molecule.

It is to the Y branches containing the variable domains harboring the antigenic epitopes to which the particular attention is directed as a predicate of the present invention.

Prior researchers have studied and manipulated entire CDRs of immunoglobulins, producing chimeric molecules that have reported functionality. Exemplary attention is directed to Jones, et al., Nature 321, 522 (1986) reporting on a V-region mouse-human chimeric immunoglobulin molecule. This research thus amounted to a substantially entire CDR replacement as apparently does the research reported by Verhoeven, et al., Science 239, 1534 (1988); Riechmann, et al., Nature 332, 323 (1988); and by Morrison, Science 229, 1202 (1985). See also European Patent Application Publication No. 125023A, published 14 November 1984.

Bolstered by the successful research summarized above that resulted presumably in functional chimeric molecules, the goal of the present research was to explore further the variable region contained in the N-terminus Y branches. It was a goal of the present research to manipulate these variable regions by introduction or substitution of novel determinants or epitopes so as to create novel immunoglobulin molecules that would possibly retain the

localization functionality and yet contain functional heterologous epitopes. In this manner, the novel immunoglobulin molecules hereof could be employed for use within the organism at foreign sites, thereby imparting
5 immunity characteristics in a novel site-directed manner.

A problem facing the present researchers at that time lay in the fact that epitopes are found in a region of the Y branch. Therefore, it was difficult to envision whether any manipulation of the variable region would be possible
10 without disrupting the interaction of heavy chain with the corresponding light chain, and if that proved inconsequential, whether the resultant molecule would retain its functionality, with respect to the novel epitope, in combination with the constant region of the
15 basic immunoglobulin molecule. Thus, even hurdling the problem of where to experiment, it was not possible to predict whether one could successfully produce such novel, bifunctional immunoglobulin molecules.

The present research and invention are based upon the
20 successful threshold experiment, producing model, novel immunoglobulin molecules found to be fully functional by virtue of their ability to localize on certain cell/receptor sites and elicit reactivity to the antigens specific for the introduced novel antigenic determinant or
25 epitope.

Summary of the Invention

The present invention is based upon the successful production of novel immunoglobulin molecules having introduced into the N-terminus variable region thereof a
30 novel epitope not ordinarily found in the immunoglobulin molecule used as a starting molecule.

Expression of oligopeptide epitopes in the hypervariable loops of an antibody molecule, antigenization of antibody,

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is an efficient procedure for stabilizing oligopeptides within a limited spectrum of tertiary structures. As a result, peptides acquire an ordered conformation, and antigenized antibodies (⁴⁸Ab) can serve as useful mimics
5 of antigens and ligands.

More particularly, this invention relates to the introduction of oligopeptide epitopes of biological receptor, preferably herein the CD4 receptor, for expression within the three-dimensional fold of an
10 immunoglobulin (Ig) molecule, thus creating molecules useful to induce specific, biologically active anti-receptor immunity.

The present invention is thus directed to novel immunoglobulin molecules having at least one novel
15 heterologous epitope contained within the N-terminus variable domain thereof, said novel immunoglobulin molecule having retained functionality with respect to its C-terminus constant domain of the heavy chain specific for a particular cell/receptor type, and having novel,
20 specific epitope *in vitro* and *in vivo* reactivity.

The present invention is further directed to pharmaceutical compositions containing, as essential pharmaceutical principle, a novel immunoglobulin hereof, particularly those in the form of an administrable
25 pharmaceutical vaccine.

The present invention is further directed to methods useful for building tolerance to certain antigens, including those associated with autoimmune diseases, or for down-regulating hypersensitivity to allergens, or for
30 providing active or passive immunity against certain pathogenic antigens, by administering to an individual in perceived need of such, a novel immunoglobulin molecule as defined above.

The present invention is further directed to novel recombinant means and methods useful for preparing, identifying and using the novel immunoglobulin molecules hereof including DNA isolates encoding them, vectors
5 operatively harboring such DNA, hosts transfected with such vectors, cultures containing such growing hosts and the methods useful for preparing all of the above recombinant aspects.

The new approach to generate protective immunity,
10 antigenized antibodies, aims at interfering with the mechanisms of virus binding and cell adhesion. ⁴⁸Abs is a new way to express immunologically-relevant oligopeptides based on the exploitation of hypervariable (HV) loops as a privileged site for the maintenance of conformation and
15 three-dimensionality. HV loops are convex sites with the ability to provide contact bonds of the ionic, hydrogen, and van der Waals types for receptors, ligands and antibodies.

The use of ⁴⁸Abs is an excellent vehicle for immunization,
20 possibly due to its intrinsic ability to conserve and/or confer tertiary structure to oligopeptides, an important feature to antigenicity and immunogenicity.

Among the advantages over conventional approaches, ⁴⁸Abs combines molecular specificity (amino acid sequence) with
25 three-dimensionality (the Ig-fold)-characteristics of key importance to antigenicity and immunogenicity - and the ability to target the target antigen presenting cells directly (APCs) via the Fc portion of the molecule. In our hands, ⁴⁸Abs elicits immunity against the native
30 antigen even across the boundaries of major histocompatibility complex (MHC) restriction.

It should be pointed out that although the emphasis of this application will be on active immunization, monoclonal antibodies of novel specificity and exquisite

biological function that may be generated in the course of these studies may be considered for passive therapy as well to complement active immunization.

Detailed Description of the Invention

5 The present invention is described herein with particular detail for the preparation of model, novel immunoglobulin entities. This description is provided, as it was conducted, using recombinant DNA technology. Further detail herein defines methods by which one can test a
10 given immunoglobulin to assure that it exhibits requisite functionality common to its starting material immunoglobulin and specially as to its novel epitopic antigenic activity. Given this information with respect to the particular novel immunoglobulin molecules described
15 herein, coupled with general procedures and techniques known in the art, the art skilled will well enough know how to configure recombinant expression vectors for the preparation of other novel immunoglobulin molecules falling within the general scope hereof for use as herein
20 described. Thus, having described the threshold experiment of the successful preparation of a novel immunoglobulin molecule, one skilled in the art need not follow the exact details used for reproducing the invention. Instead, the art skilled may borrow from the
25 extant, relevant art, known techniques for the preparation of still other novel immunoglobulin molecules falling within the general scope hereof.

1. Figure Legends

Figure 1 is a diagram illustrating the construction of the
30 pNy1NANP expression vector.

Figure 2 is an SDS-PAGE of the y1NANP and WT recombinant Ig.

Figure 3 shows the binding of ^{125}I -labelled monoclonal antibody Sp-3-B4 to engineered antibody y1NANP.

Figure 4 is a Western blot binding of ^{125}I -labelled antibody Sp3-B4 to engineered antibody y1NANP and
5 localization of the engineered (NANP), epitope in the H chain.

Figure 5 shows results of cross-inhibition of ^{125}I -labelled antibody Sp3-B4 binding to synthetic peptide (NANP),
(panel A) or engineered antibody y1NANP (panel B) by
10 y1NANP Ig or peptide (NANP),.

Figure 6 is a stereo drawing of the α carbon backbone of the first (V1) extracellular domain of human CD4 (residues 1-98) from the x-ray crystal analysis.

Figure 7 depicts structure and epitope expression of ^{125}I -Abs
15 expressing oligopeptides of human CD4 and reactivity with an anti-CD4 antibody.

Figure 8 shows C57BL/6 mice immunized with $\gamma 1\text{CD4}^{\text{B}}$ (100 μg /injection) intraperitoneally at monthly intervals first in complete Freund's adjuvant and subsequently in
20 incomplete adjuvant. Hybridomas were prepared from a mouse with the highest titer of inhibitory antibodies by fusing with Sp2/0 myeloma cells. Syncytia assay conditions and percent inhibition were described in the text.

25 Figure 9 provides an analysis of mAbPL1. Upper panel: Dose-response inhibition of syncytia formation. Lower panel: FACS analysis and surface staining of CD4 on CEM cells.

Figure 10 is a Western Blot on recombinant (γ)CD4. 2 μg
30 of soluble rCD4 were loaded onto a 10% SDS/PAGE gel, electrophoresed and transferred to PVDF membrane

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(Millipore). After blotting, the membrane was blocked by soaking in 5% dry milk in PBS and incubated with 10 μ g/ml of each mAbPL overnight at 4 °C. Binding was revealed with a HRP-conjugated goat antibody to mouse Ig (γ -chain specific) in PBSA. The OKT4D and a noncorrelate Ab have been used as a positive and negative control, respectively.

2. General Methods and Definitions

"Expression vector" includes vectors which are capable of expressing DNA sequences contained therein, where such sequences are operatively linked to other sequences capable of effecting their expression. It is implied, although not always explicitly stated, that these expression vectors may be replicable in the host organisms either as episomes or as an integral part of the chromosomal DNA. "Operative," or grammatical equivalents, means that the respective DNA sequences are operational, that is, work for their intended purposes. In sum, "expression vector" is given a functional definition, and any DNA sequence which is capable of effecting expression of a specified DNA sequence disposed therein is included in this term as it is applied to the specified sequence. In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" referred to as circular double stranded DNA loops which, in their vector form, are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

Apart from the novelty of the present invention involving the introduction of novel epitopes by means of repositioning or augmentation of a parent immunoglobulin,

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it will be understood that the novel immunoglobulins of the present invention may otherwise permissively differ from the parent in respect of a difference in one or more amino acids from the parent entity, insofar as such
5 differences do not lead to a destruction in kind of the basic activity or bio-functionality of the novel entity.

"Recombinant host cells" refers to cells which have been transfected with vectors defined above.

Extrinsic support medium is used to support the host cells
10 and includes those known or devised media that can support the cells in a growth phase or maintain them in a viable state such that they can perform their recombinantly harnessed function. See, for example, ATCC Media Handbook, Ed. Cote et al., American Type Culture
15 Collection, Rockville, MD (1984). A growth supporting medium for mammalian cells, for example, preferably contains a serum supplement such as fetal calf serum or other supplementing component commonly used to facilitate cell growth and division such as hydrolysates of animal
20 meat or milk, tissue or organ extracts, macerated clots or their extracts, and so forth. Other suitable medium components include, for example, transferrin, insulin and various metals.

The vectors and methods disclosed herein are suitable for
25 use in host cells over a wide range of prokaryotic and eukaryotic organisms.

"Heterologous" with reference herein to the novel epitope for a given immunoglobulin molecule refers to the presence of (at least one) such epitope in the N-terminus domain of
30 an immunoglobulin that does not ordinarily bear that epitope(s) in its native state. Hence, that chain contains heterologous epitope sequence(s). Such heterologous epitope sequences shall include the classic antigenic epitopes as well as receptor binding domains or binding

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regions that function as receptor sites, such as the human CD4 binding domain for HIV, hormonal receptor binding ligands, retinoid receptor binding ligands and ligands or receptors that mediate cell adhesion.

- 5 "Chimeric" refers to immunoglobulins hereof, bearing the heterologous epitope(s), that otherwise may be composed of parts taken from immunoglobulins of more than one species. Hence, a chimeric starting immunoglobulin hereof may have a hybrid heavy chain made up of parts taken from
10 corresponding human and non-human immunoglobulins.

In addition to the above discussion and the various references to existing literature teachings, reference is made to standard textbooks of molecular biology that contain definitions and methods and means for carrying out
15 basic techniques encompassed by the present invention. See, for example, Maniatis, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1982 and the various references cited therein, and in particular, Colowick et al., Methods in Enzymology Vol
20 152, Academic Press, Inc. (1987). All of the herein cited publications are by this reference hereby expressly incorporated herein.

The foregoing description and following experimental details set forth the methodology employed initially by
25 the present researchers in identifying and characterizing and preparing particular immunoglobulins. The art skilled will recognize that by supplying the present information including the wherewithal of the location and makeup of the epitope containing domain of a given immunoglobulin,
30 and how it can be manipulated to produce the novel immunoglobulins hereof. Therefore, it may not be necessary to repeat these details in all respects in their endeavors to reproduce this work. Instead, they may choose to employ alternative, reliable and known methods,
35 for example, they may synthesize the underlying DNA

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sequences encoding a particular novel immunoglobulin hereof for deployment within similar or other suitable, operative expression vectors and culture systems. Thus, in addition to supplying details actually employed, the present disclosure serves to enable reproduction of the specific immunoglobulins disclosed and others, and fragments thereof, such as the individual chains for *in vitro* assembly, using means within the skill of the art having benefit of the present disclosure. All of such means are included within the enablement and scope of the present invention.

3. Description of Particularly Preferred Embodiments

Research focused on the engineering, expression and testing of antibodies containing discrete sequences of human CD4. The four regions used for "antigenization" are listed below (Table I) and are also depicted in Figure 6 in the context of three-dimensional folding of CD4. The first two sequences used correspond to the putative HIV-binding site were 42-49 and 41-55. The two new sequences correspond to amino acid residues 38-49. Among the latter, one is the wild type (WT) sequence, the other contains a mutation glutamine (Q) to alanine (A) in position 40 that according to published data (24) possesses significantly increased affinity for gp120. In each instance, these oligopeptide sequences were inserted in the CDR3 loop of a murine heavy chain V region gene expressed along with a human $\gamma 1$ constant region gene to yield a chimeric (mouse/human) H chain. The insertion of the CD4 peptides was confirmed by nucleotide sequence analysis.

TABLE I

5	A	SFLT	KGPS	
	B	GSFLT	KGPSK	LNDRA
	C	GNQ	GSFLT	KGPS
	D	GNA	GSFLT	KGPS
		↑		↑
		38		55 of CD4

10 4. Examples

The engineering steps were as follows, with reference made to Figure 7. a) General structure of pNylCD4 expression vector modified to encode peptides 42-49^(A) or 41-55^(B) of human CD4. The CDR3 region of the host V_H (KAYSHG; residues 93-98) was mutagenized to introduce a single KpnI/Asp718 site and yield the intermediate sequence KVPYSHG; residues 93-99. Here the amino acid 94A was deleted and substituted with the VP doublet which is encoded in the Asp718 cloning site. Subsequently, complementary oligonucleotides coding for peptides 42-49 or 41-55 of human CD4 sequence were introduced between 94V and 95P of the mutagenized V_H region. Only the coding oligonucleotide strands are shown here. The engineered V_HCD4^A or V_HCD4^B coded by the 2.3-kilobase (kb) EcoRI fragment was cloned upstream from a human γ 1 constant (C) region gene contained in the 12.8 kb vector pNyl. [Solazzo et al., Focus 10, 64 (1988). This is a PSV vector harboring a human γ 1 gene, encoded downstream from the EcoRI site. It also carries a neomycin resistance gene under the control of the SV40 promoter for the selection of stable transformant cells. See also Solazzo et al., Eur. J. Immunol. 19, 453 (1989).]

About 30 μ g of the final DNA constructs pNylCD4^A or pNylCD4^B were electroporated in the murine J558L cell line (20 x 10⁶ cells) using a field strength of 750 V/cm. Transfected cells were incubated without selection for 24

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h and then selected in presence of 1.2 mg/ml G418 (GIBCO). G418-resistant clones secreting high level of the ¹²⁵I-Abs were identified by enzyme-linked immunosorbent assay (ELISA) using horseradish peroxidase (HRP)-conjugated goat antibody to human immunoglobulin (Ig) (SIGMA).

¹²⁵I-Abs were first concentrated by (NH₄)₂SO₄ precipitation and then purified by affinity chromatography on a Protein A (Pharmacia-LKB, Alameda, CA) column equilibrated with 3M NaCl/1M glycine, pH 8.9. Elution was performed using Glycine 0.1M- HCl/0.5M NaCl pH 2.8. The eluted fractions were neutralized using 1M Tris-HCl pH 8.0 and dialysed against phosphate-buffered saline (PBS). B, BamHI; RI, EcoRI; Neo, neomycin (G418) resistance; Amp, ampicillin resistance.

b) Schematic view of ¹²⁵I-Abs expressing CD4 peptides. The heavy chain is the fusion product of a human γ 1C region with a murine V_H region engineered to express the CD4 sequences 42-49 and 41-55. The CDR3 region of the H chain was modified by inserting between 94V and 95P the residues SFLTKGPS(upper)^(A) or GSFLTKGPSKLNDR (lower)^(B) sequences. The inserted sequences are flanked at each side by a VP doublet. The λ , light (L) chain is provided by the murine myeloma J558L used for transfection.

c) Recognition of the two ¹²⁵I-Abs by OKT4D a monoclonal antibody to CD4. ¹²⁵I-Abs were electrophored on a 10% Sodium dodecyl Sulfate (SDS)/Polyacrylamide Gel (PAGE) and 1.2 μ g per lane were run under reducing conditions (5% β -mercaptoethanol). Protein were blotted onto 0.45- μ m Polyvinylidene difluoride paper (PVDF) (Millipore). after blotting, the membrane was blocked by soaking in 5% dry milk in PBS and incubated for 1 hour at room temperature with OKT4D (2.5 μ g/ml), Pharmaceutical Research Institute-Raritan-NJ) a murine monoclonal antibody (IgG1,k) that binds residues 44-52 of human CD4. The bound antibody was revealed by ¹²⁵I-labeled goat antibody to mouse k. PVDF

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paper was exposed to Kodak X-OMAT AR film for 3 days at -70 °C. A band in correspondence of the heavy chain is visible on the ^{125}I -Ab $\gamma 1\text{CD4}^A$ and ^{125}I -Ab $\gamma 1\text{CD4}^B$ but not in the $\gamma 1\text{WT}$ control.

- 5 The antigenized antibodies produced were expressed as transfectoma products obtained by introducing the appropriate plasmid vector (containing the engineered V domain) into J558L mouse myeloma cell line by electroporation. This cell line is a H chain-defective
10 variant of myeloma J558, and carries the rearrangement for a $\lambda 1$ light (L) chain. The overall structure of the antibodies that are obtained by this procedure is depicted in Figure 7. The supernatants of neomycin resistant colonies (stable transformants have been tested by enzyme-
15 linked immunosorbent assay (ELISA) for immunoglobulin (Ig) production using goat antibodies to human Ig in a sandwich assay.

- By this method, we selected cultures secreting $\geq 20 \mu\text{g/ml}$. ^{125}I -Abs have been purified from culture supernatants by
20 affinity chromatography on Protein A-Sepharose. The purified proteins have been concentrated and analyzed by SDS-PAGE for purity using Coomassie blue staining. Verification of the expression of the CD4 inserts has been done by solid phase radioimmunoassay (RIA) and Western
25 blot using OKT4D, a monoclonal antibody to CD4 whose recognition site is around amino acid residue 47. Table II indicates that the four ^{125}I -Abs^{CD4} all bound in a dose dependent manner glycosylated, recombinant gp120 in a solid-phase assay, hence suggesting that the residues
30 grafted into the host antibody molecule are sufficient to mediate binding. A reference HIV⁺ serum was used as a positive control.

TABLE II

Antibodies Antigenized with Peptide Structures of
Human CD4 Bind Recombinant gp120 in ELISA

Antibody	CD4 Residues		ELISA O.D. (A_{492})		
			100 ($\mu\text{g/ml}$)	25 ($\mu\text{g/ml}$)	6.25 ($\mu\text{g/ml}$)
5	γ 1CD4	A 42-49	1435	976	686
	"	B 41-55	1124	722	541
	"	C 38-49	1401	1110	817
	"	D 38-49 ^{Q10A}	1137	1093	962
	γ 1WT		618	496	476
10	HIV ⁺ serum (1:1000)		1283		
	HIV ⁻ serum (1:1000)		525		

The first two γ 1Abs^{CD4} were tested for their ability to induce site-specific and biologically-active antibodies to CD4. In particular, we intended to generate antibodies
15 against the HIV interactive site of CD4 that could block syncytia formation. Precisely, we used a rapid method for the formation and analysis of syncytia *in vitro*. This test utilizes CD4⁺ 8E5 T cells (transfected with a HIV defective for reverse transcriptase) and CD4⁺ human T
20 cells MOLT3. When these two cells are incubated for 3 hours at 37°C, syncytia form in large number and these can be quantified by visual inspection.

8E5 ($1.2 \times 10^6/\text{ml}$) and MOLT3 ($2 \times 10^6/\text{ml}$) cells were mixed in equal volumes in a final volume of 300 μl , plated in 96-
25 well plate and incubated at 37°C for 30 minutes. The plate was then spun for 5 minutes at 1200 rpm and incubated at 37°C for an additional 3 hours. At the end of the incubation the cells were transferred to a flat-bottom 96-well plate and the formation of syncytia
30 recorded.

Rabbits were immunized at regular intervals for a total of 5 injections. Sera were taken 10 days after the

immunizing injection. Only rabbits immunized with ^{125}I AbCD4^B (residues 41-55) produced syncytia-inhibiting antibodies (Figure 7). By FACS, 2 out of 5 bound, to some extent, to CD4. CD4 spontaneously binds Ig of unrelated specificity, thereby making it difficult to discriminate between paratope-dependent and paratope-independent binding. See Table III.

TABLE III

NZW Rabbits
100 µg/injection
x5 subcutaneously

10

15

20

25

Rabbit #	Immunogen	FACS (CEM cells)	Syncytia Inhibition (%) **
1	$\gamma 1\text{CD4}^{42-49}$	-	5
2	"	-	0
3	"	-	0
4	"	-	0
5	"	-	8
6	$\gamma 1\text{CD4}^{41-55}$	+	45
7	"	+	28
8	"	-	30
9	"	-	35
10	"	-	0

* sera were used at the dilution of 1:500

** Inhibition was evaluated as follows:

% of inhibition postimmune serum - % of inhibition
preimmune serum

Similarly, mice were immunized using only ^{125}I AbCD4^B and obtained syncytia inhibiting antibodies in every instance. From a mouse with the highest titer (1:500) several hybridomas were generated and screened using the microsyncytia assay. Out of many positive clones, we retained four (Figure 8). All were of the IgG1, k isotype and inhibited when diluted 1:2 with values ranging from 45 to 82 %. The 4 clones were further subcloned, purified and characterized. As an example, we have displayed the results obtained with one of them (mAbPL1) (Figure 9).

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The upper panel shows a dose-dependent effect of this antibody on syncytia formation. In the lower panel, a FACS profile on CEM cells is shown using mAbPL1 and by comparison Leu3a. Antibody PL1 bound with a unimodal peak, but with intensity lower than Leu3a. All antibodies also bound with CD4 in Western blot and the immunizing ¹²⁵IAb (Figure 9).

Taken together, these results indicate that ¹²⁵IAb^{CD4-11} efficiently elicited antibodies against the site of CD4 engineered into the Ig V_H domain, further indicating that the conformation retained by the CD4 14^{mer} integrated within the Ig loop is adequate for immunogenicity. Thus, this method appears to provide an ideal solution to the limitation of immunogenicity of synthetic peptides.

15 5. References

The following references are grouped by number referring to parenthetical numbering in the preceding text. Each of these documents is hereby expressly incorporated by reference herein:

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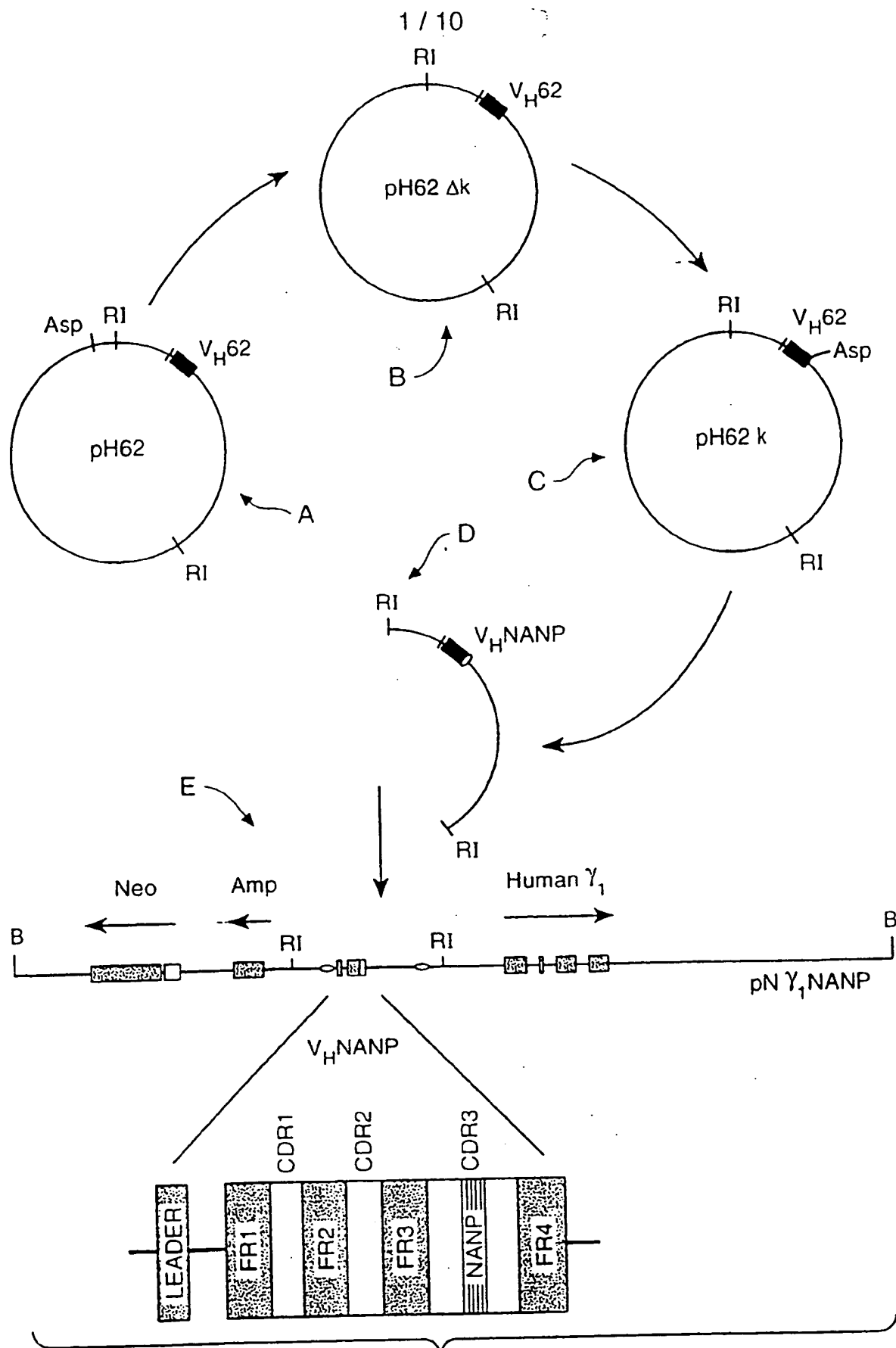
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- 10 The foregoing description details specific methods that can be employed to practice the present invention. Having detailed specific methods initially used to identify, isolate, characterize, prepare and use the immunoglobulins hereof, and a further disclosure as to specific model
- 15 entities, the art skilled will well enough know how to devise alternative reliable methods for arriving at the same information and for extending this information to other intraspecies and interspecies related immunoglobulins. Thus, however detailed the foregoing may
- 20 appear in text, it should not be construed as limiting the overall scope hereof; rather, the ambit of the present invention is to be governed only by the lawful construction of the appended claims.

Claims:

1. An immunoglobulin molecule containing at least one CD4 HIV binding domain within the third complementarity-determining region (CDR) in the N-terminus variable domain thereof, said immunoglobulin molecule having the effector function conferred by the constant region of the immunoglobulin, and having specific CD4 epitope reactivity.
2. An immunoglobulin according to Claim 1 wherein said binding domain is the region of amino acid residues selected from the group consisting of 42 to 49, 41 to 55, and 38 to 49.
3. As a product of recombinant DNA technology, an immunoglobulin according to Claim 1.
4. A heavy chain of an immunoglobulin containing within the third complementarity-determining region (CDR) in the N-terminus variable domain thereof at least one CD4 HIV binding domain.
5. As a product of recombinant DNA technology, the heavy chain according to Claim 3.
6. The heavy chain according to Claim 4 in a form unassembled with its counterpart heavy chain.
7. The heavy chain according to Claim 6 in a form unassembled with its associated light chain.
8. A chimeric immunoglobulin molecule according to Claim 1.
9. The chimeric immunoglobulin molecule according to Claim 7 comprising hybrid heavy chain comprising both human and non-human sequences.

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10. A pharmaceutical composition containing as an essential principle an immunoglobulin molecule according to Claim 1.
11. The composition according to Claim 10 suitable for administration to a human subject.
12. The composition according to Claim 10 in the form of an administrable vaccine.
13. A DNA molecule that is a recombinant DNA molecule or a cDNA molecule encoding an immunoglobulin molecule according to Claim 1.
14. An expression vector operatively harboring DNA encoding an immunoglobulin, defined according to Claim 13.
15. A recombinant host cell transfected with an expression vector according to Claim 14.
16. A process of preparing an immunoglobulin molecule according to Claim 1 which comprises expressing in a recombinant host cell transfecting DNA encoding said immunoglobulin molecule.

**FIG. 1A**

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CDR3

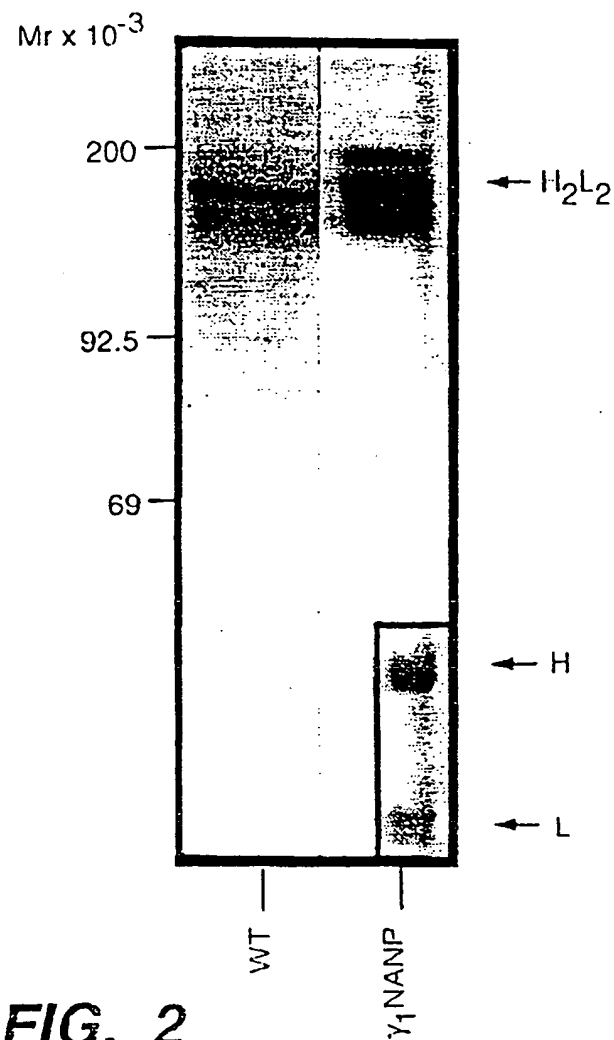
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pH NANP

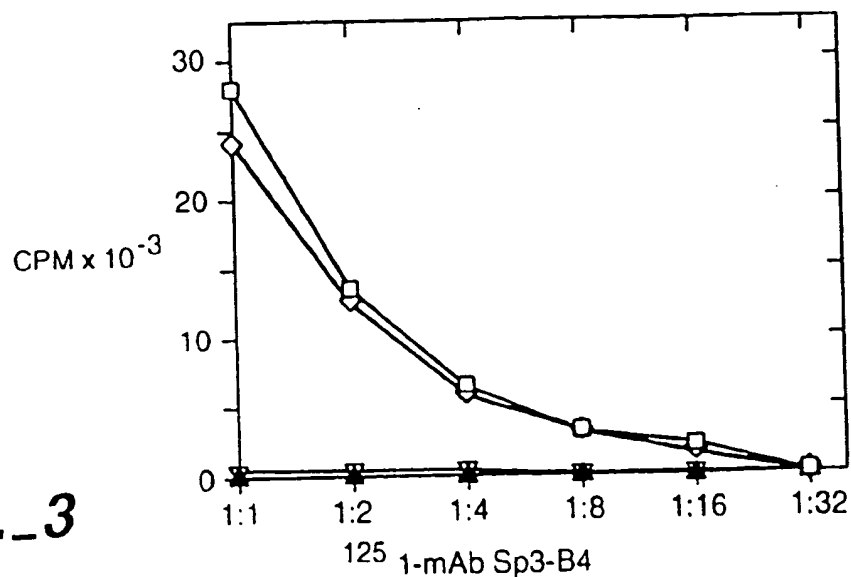
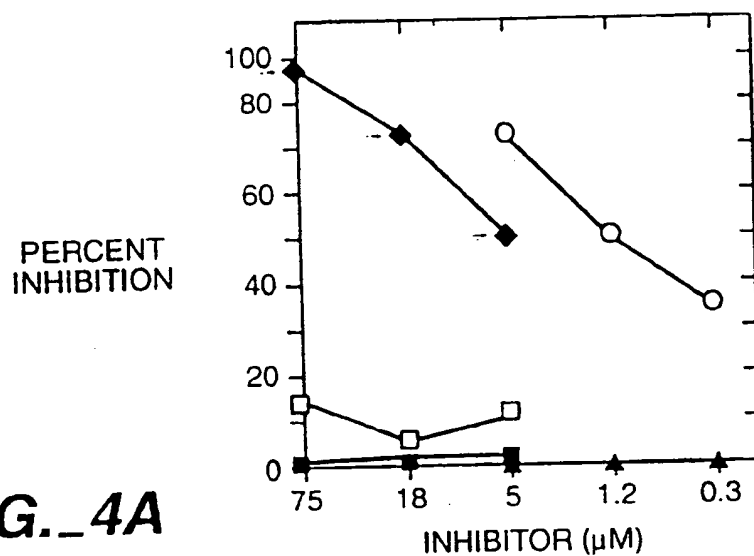
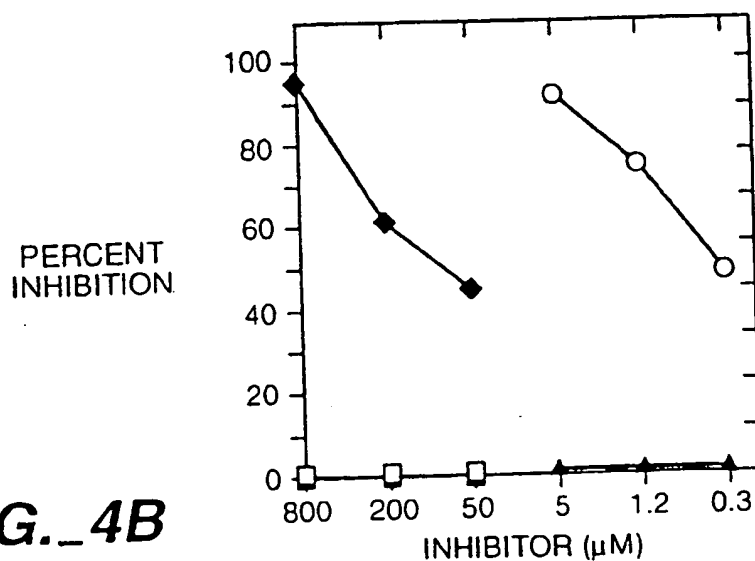
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FIG. 1B

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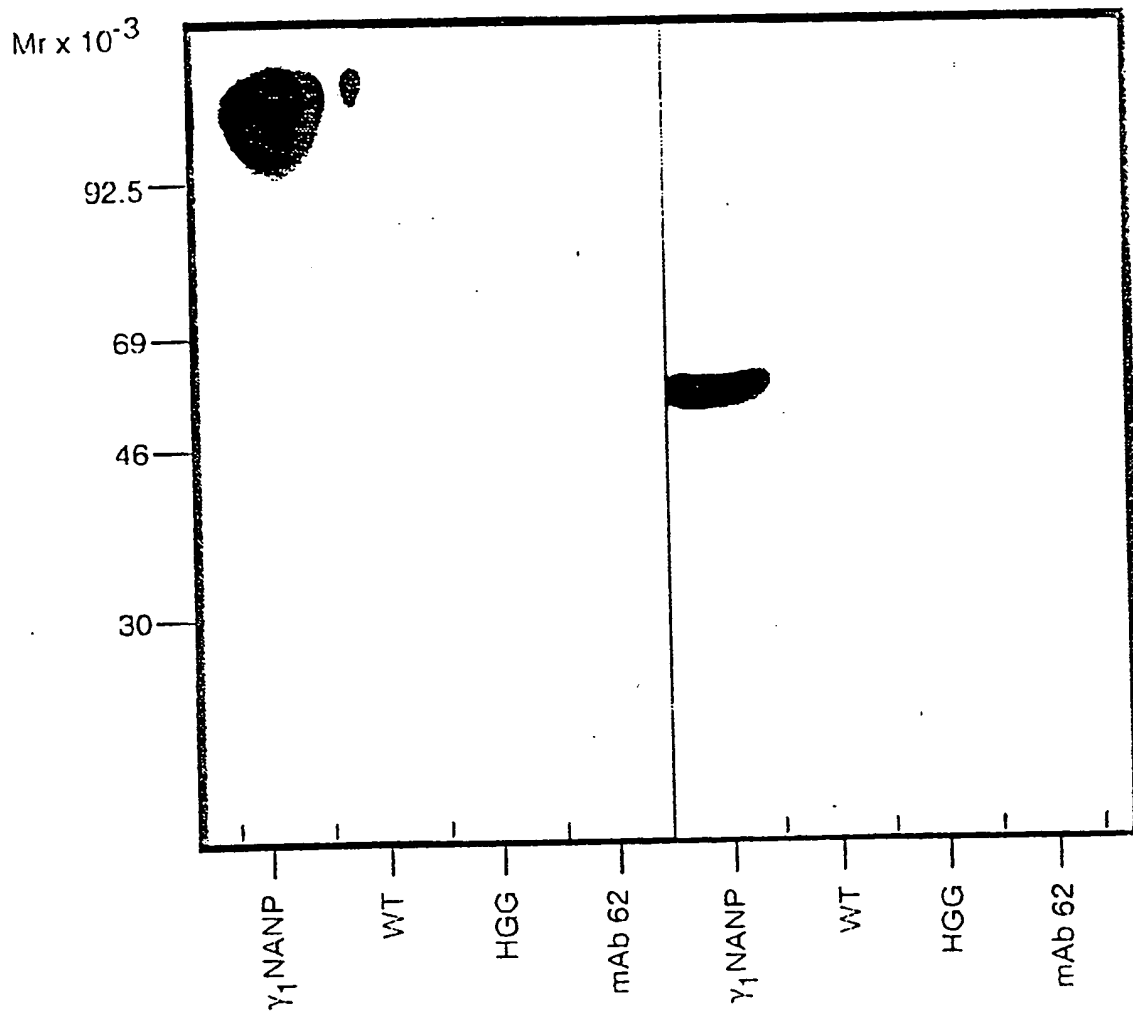
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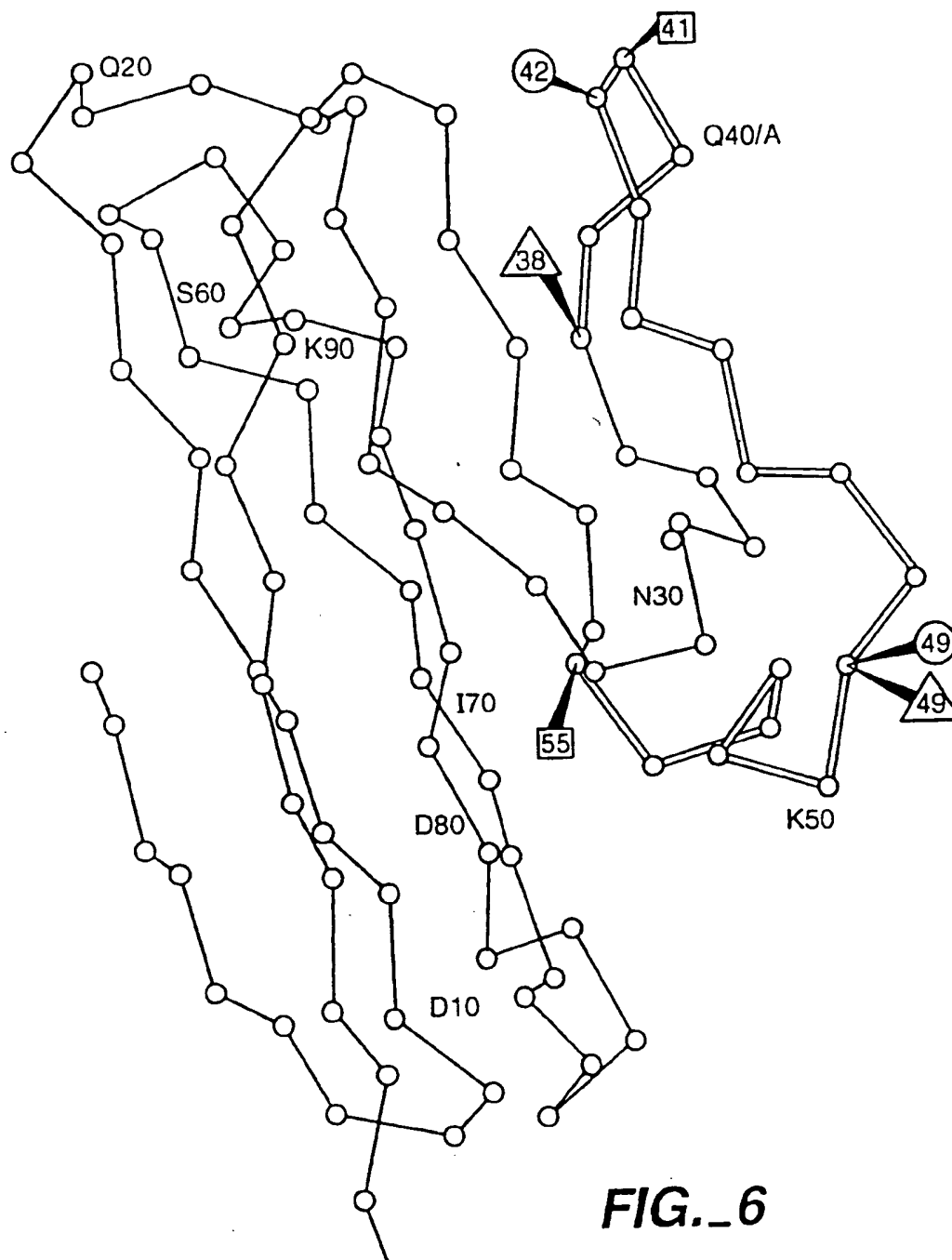
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FIG._3**FIG._4A****FIG._4B**

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**FIG. 5**



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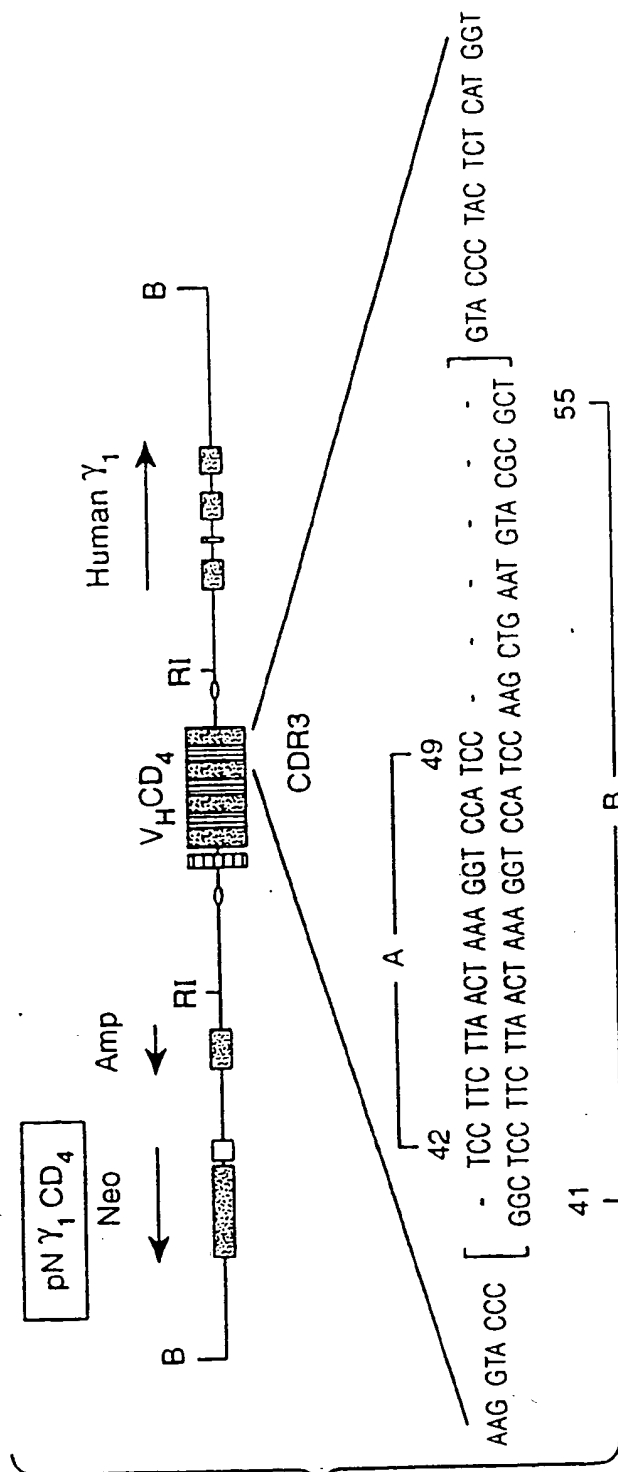


FIG. 7A

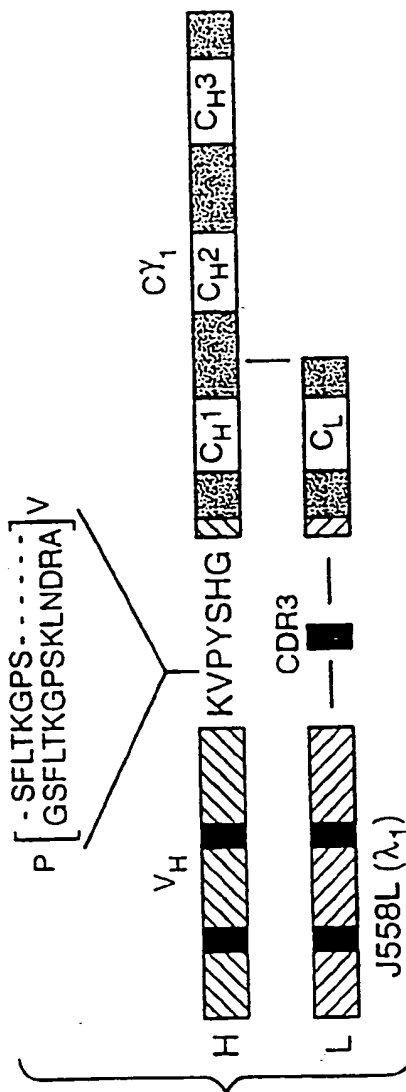
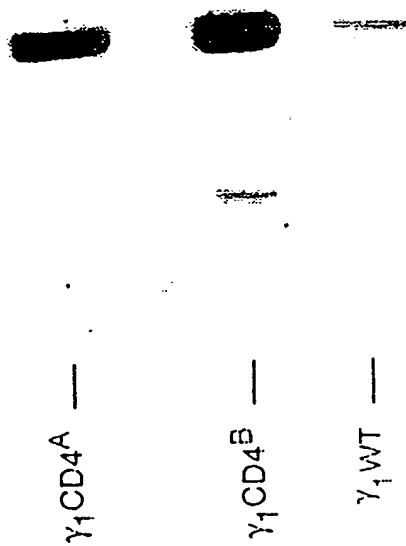
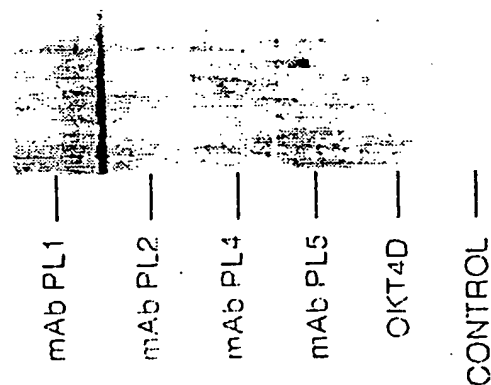


FIG. 7B

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**FIG. 7C****FIG. 10**

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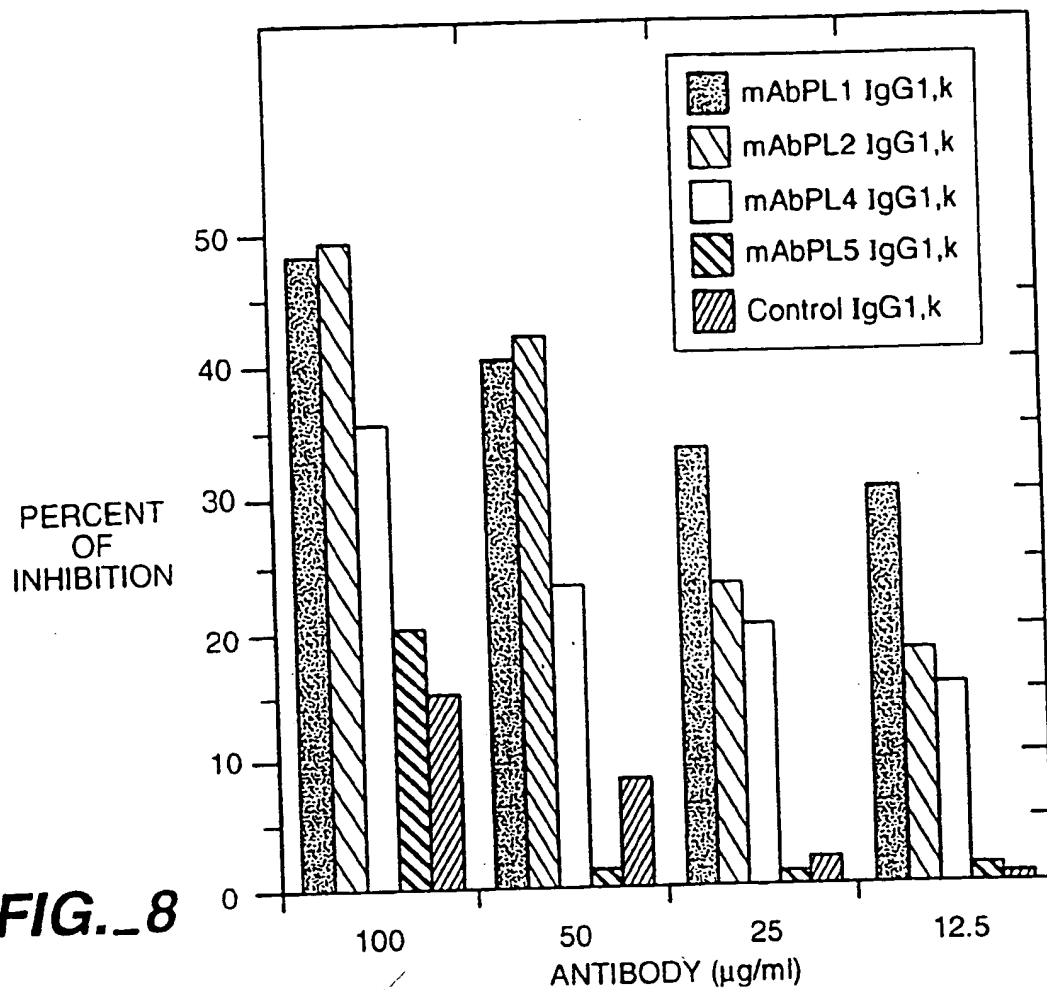


FIG. 8

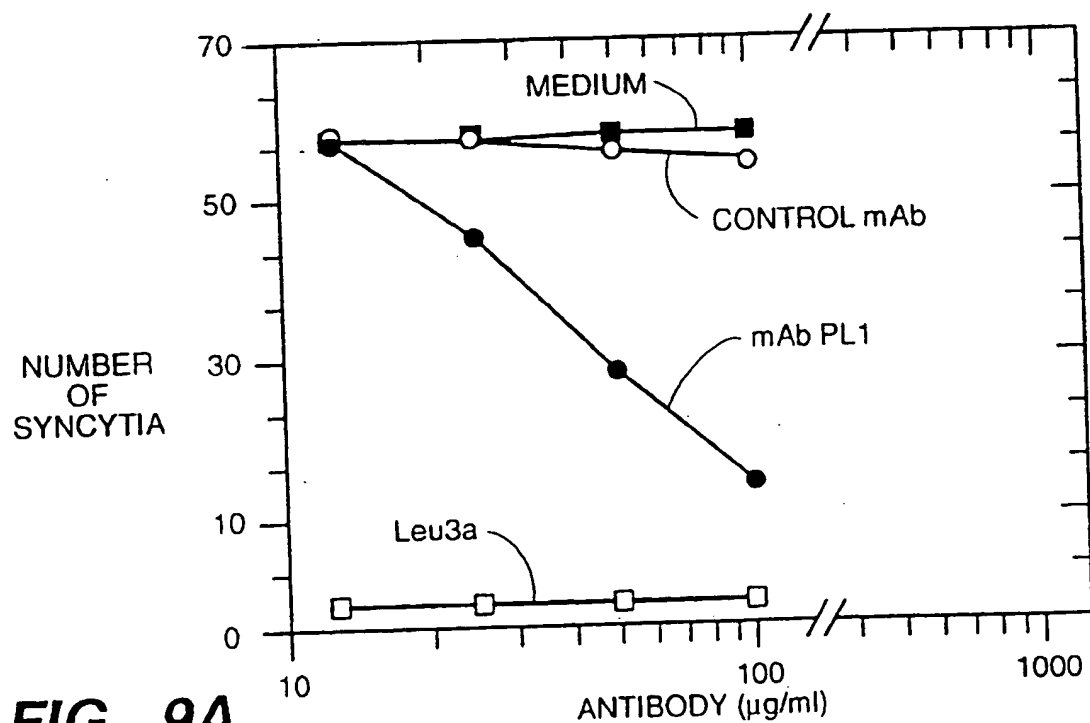
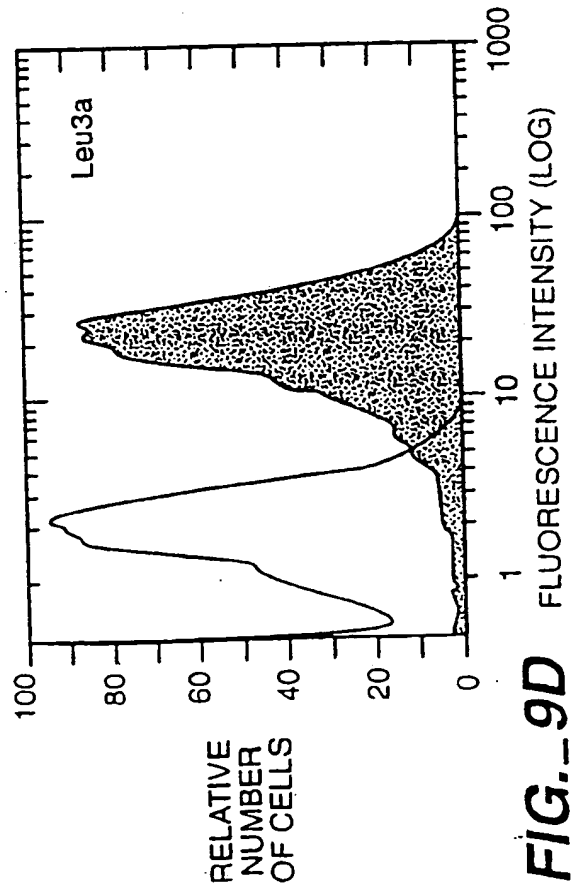
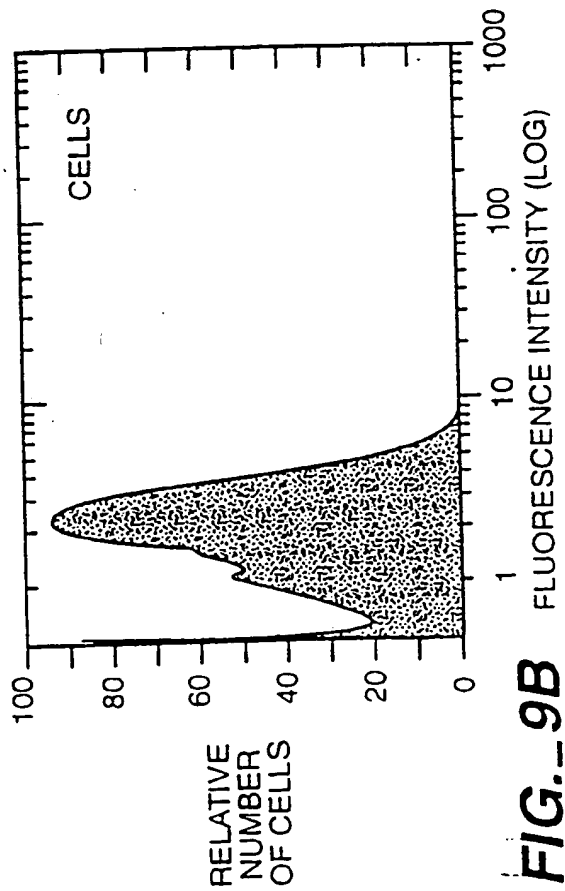
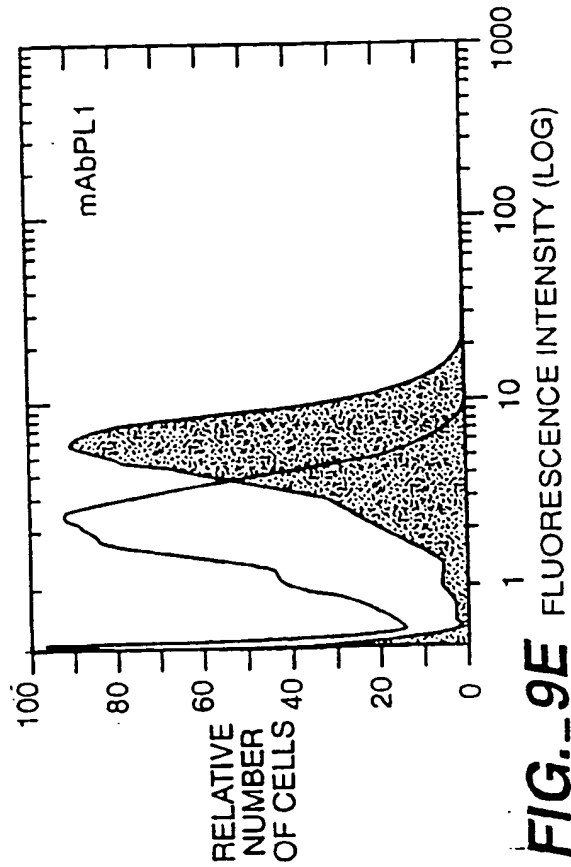
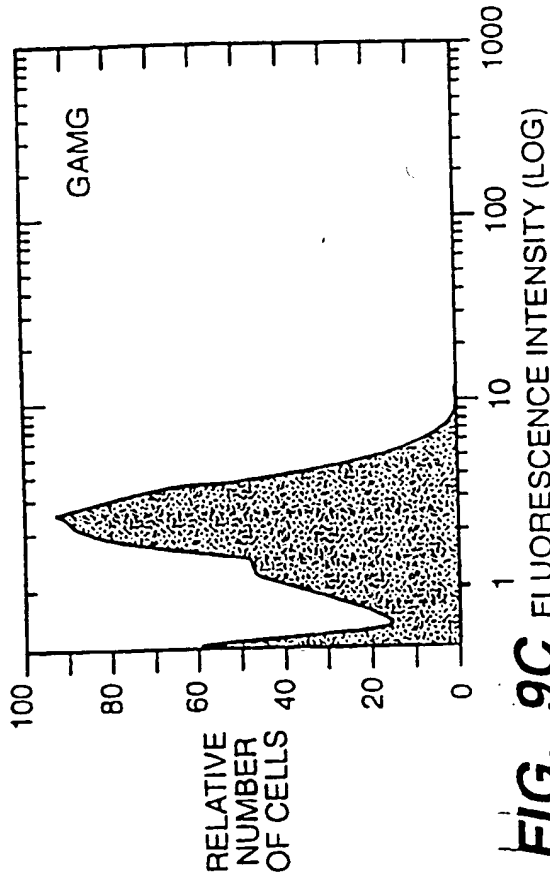


FIG. 9A

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A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : Please See Extra Sheet.

US CL : 530/387.3; 536/25.23; 435/69.6, 320.1; 424/85.8.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/387.3; 536/25.23; 435/69.6, 320.1; 424/85.8.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences, Volume 88, issued June 1991, Billetta et. al. "Immunogenicity of an Engineered Internal Image Antibody", pages 4713-4717, see especially pages 4713-4715.	1-16
Y	The Journal of Biological Chemistry, Volume 266, No. 9, issued 25 March 1991, Truneh et. al., "A Region in Domain 1 of CD4 Distinct From the Primary gp120 Binding Site Is Involved In HIV Infection and Virus-mediated Fusion*", pages 5942-5948, see entire article.	1-16

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be part of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

31 MARCH 1994

Date of mailing of the international search report

19 APR 1994

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	The FASEB Journal, Volume 6, No.5, issued 28 February 1992, Billetta et. al, "Immunogenicity of Hydrophylic Sequences In Antegenized Antibodies", No. 6226, see entire abstract.	1-16
Y	Immunomethods, Volume 1, issued 1992, Billetta et. al., "Ligand Expression Using Antigenization of Antibody: Principle and Methods", pages 41-51, see especially pages 44-48.	1-16

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (5):

C07K 15/28; C12P 21/06; A61K 39/395; C12N 15/00, 1/00, 5/00; C07H 15/00.